IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

ATTORNEY DOCKET NO. 30523/105/ANRC

In re patent application of

Henryk LUBON *et al.*

U.S. Serial No. 07/943,246 Group Art Unit: 1804

Filed: September 10, 1992 Examiner: D. Crouch

For: EXPRESSION OF ACTIVE HUMAN PROTEIN C IN MAMMARY TISSUE OF TRANSGENIC ANIMALS USING A LONG WAP PROMOTER

DECLARATION UNDER 37 CFR §1.132 OF KENNETH R. BONDIOLI

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Kenneth R. Bondioli, state and declare that:

1. I am a staff scientist employed by Alexion Pharmaceuticals, Inc., New Haven, Connecticut. My resume and list of publications is appended hereto as Exhibit A.

2. I have read and understand the specification of the above-captioned application ("the Lubon application"). In particular, I am familiar with the disclosure relating to methods for producing transgenic cattle.

3. In 1987, I participated in a transgenic cattle project as an employee of Granada BioSciences, Inc. (College Station, Texas). The "major breakthrough" which made the production of transgenic cattle possible at that time was the technique of centrifuging zygotes to allow visualization of pronuclei. This method was published by Wall *et al., Biol. Reprod. 32*: 645-651 (1987), and it is described in the Lubon application.

4. The transgenic cattle project was motivated by an interest in demonstrating that transgenic cattle could be

produced by the method of pronuclear injection which had been demonstrated for various other species. To accomplish this goal in a timely manner, we did not allow transferred embryos to be carried to term. Instead, we collected and analyzed about 60 day-old fetuses for the presence of the transgene in fetal tissue. We briefly reported our success in producing transgenic fetuses in the publication of Biery, *et al., Theriogenology 29*: 224 (1988). See Exhibit B.

5. In December of 1988, I presented a more detailed account of our methods and results at a symposium held at the National Institutes of Health. The meeting was entitled "Symposium on Transgenic Technology in Medicine and Agriculture," and it was sponsored by the Center for Population Research, National Institute of Child Health and Human Development. My contribution to that symposium was later published as a chapter in a book which contained the complete proceedings of the meeting. See the publication of Bondioli *et al.*, "Production of Transgenic Cattle by Pronuclear Injection," in TRANSGENIC ANIMALS, First *et al.* (eds.), pages 265-273 (Butterworth-Heinemann 1991) [Exhibit C].

6. Between 1988 and 1991, we utilized these same techniques to produce live transgenic cattle with four different transgenes. Our first transgenic bovine was born in 1989, and this result was reported at a symposium entitled "Genetic Engineering of Animals" (Ithaca, NY; June 1989). This contribution was published in "Genetic Engineering of Animals," *J. Reprod. Fertil. Suppl. No. 41* (1990). See Exhibit D. The complete results of the bovine transgenic program at Granada BioSciences, Inc. were published in January of 1992. See Hill *et al., Theriogenology 37*: 222 (1992) [Exhibit E].

7. In summary, the scientific knowledge available as of January of 1991 in combination with the disclosure of the above-

captioned application would have enabled a researcher to produce a transgenic cow having a transgene under the control of the "long WAP promoter."

8. Finally, I understand that there has been some concern on the part of the U.S. Patent and Trademark Office regarding the relatively few scientific reports on transgenic cattle production published by the priority date of the Lubon application. I believe that the observation that there are few reports of transgenic cattle by 1991 was strictly a function of economics, and was not due to a lack of technical know-how.

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

December 16, 1996
Date

Kenneth R. Bondioli
Kenneth R. Bondioli

Exhibit A

KENNETH R. BONDIOLI

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CAREER SUMMARY

Research scientist with 13 years experience in embryo biotechnology. Specializes in development of and application of state of the art technology for commercial purposes.

RESEARCH EXPERIENCE

- Production of transgenic animals. Laboratory and livestock species.
- Embryo cloning by nuclear transplantation.
- Oocyte maturation and in vitro fertilization.
- Embryo culture.
- Embryo sexing by PCR.
- Embryo cryopreservation.

TECHNICAL SKILLS

- **Embryo Manipulation:** Pronuclear injection● In vitro maturation and fertilization of bovine oocytes●Nuclear transplantation●Embryo biopsy●Embryo culture●Embryo cryopreservation

- **Molecular Biology:** Sub cloning of DNA constructs●PCR●Southern blot●Fluorescent in situ hybridization

ADMINISTRATIVE SKILLS

Supervision of scientific and technical staff●Formulation of budgets●Development of technical prospectuses●Preparation of progress reports●Writing grant applications●Preparation of patent applications●Member institutional biosafety committee●Served as radiation safety officer

MAJOR ACCOMPLISHMENTS

- Built research department of Granada BioSciences from one scientist to a total staff of 15, including 5 scientists.

- Produced first transgenic cattle.

- Participated in the production of the first cloned cattle.

- Developed embryo sexing assay.
- Participated in the establishment and operation of a bovine oocyte in vitro maturation, fertilization and culture system in two separate laboratories.
- Prepared as an inventor four patent applications and served as scientific coordinator for the prosecution of all company patents.
- Published two book chapters, 26 articles in refereed journals and 38 abstracts.

EMPLOYMENT HISTORY

- Alexion Pharmaceuticals, Inc. Staff Scientist. November 1996- Present.
- Research Biologist with USDA, Gene Evaluation and Mapping Laboratory, Beltsville Agriculture Research Center. August 1995-August 1996.
- Altra Bio. Inc. Principal scientist, serving as visiting scientist at USDA, Gene Evaluation and Mapping Laboratory under a Cooperative Research Agreement. 1993-1995.
- American Breeders Service, Specialty Genetics Division.
  Special consultant. 1992-1993.
- Granada BioSciences, Inc. Senior research scientist. 1982-1992.

EDUCATION

- Washington State University, Ph.D., 1982
  Thesis Topic: In vitro fertilization of ovine and bovine oocytes.
- Washington State University, M.S., 1979
  Thesis Topic: Superovulation of ewes with gonadotrophins and in vitro fertilization of ovulated oocytes.
- Cornell University, B.S. (Animal Science), 1973

PROFESSIONAL ASSOCIATIONS

- Society for the Study of Reproduction
- American Society of Animal Science
- International Embryo Transfer Society
  President 1992
  Vice President 1991
  Member Board of Directors 1990-1991, 1992-1993

INVITED LECTURES

University of Connecticut, Biotechnology Center, Storrs, Connecticut. "Transgenic Livestock--State of the Art and Prospects for the Future." 1996

Genzyme Transgenic Corp. Framingham, MA. "Transgenic Cattle--State of the Art and Prospects for the Future." 1996.

PPL Therapeutics, Inc. Blacksburg, Virginia. "Selection of Transgenic Bovine Embryos in Culture." 1995.

Trans ova Genetics. Sioux Center, Iowa. "Transgenic Livestock, State of the Art and Prospects for Commercialization." 1995.

Animal Co-Products Utilization Workshop. Kansas City, Kansas. "Genetic Engineering for Animal Products." 1994.

Symposium on Recent Developments in Embryo Biotechnology for Farm Animals. Baton Rouge, Louisiana. "Embryo Cloning by Nuclear Transfer in Cattle, Present Status and Prospects for the Future." 1993.

Symposium on Potentials and Limitations of Biotechnology for Livestock Breeding and Reproduction in Developing Countries. Mariensee, Germany. "Multiplication of Cattle Embryos by Nuclear Transfer." 1992.

Symposium on Cloning Mammals by Nuclear Transplantation. Fort Collins, CO. "Commercial Cloning of Cattle by Nuclear Transfer." 1992.

Cornell University, Ithaca, NY. "Embryo Technologies in Animal Production." 1991.

International Symposium on Animal Biotechnology. Kyoto University, Kyoto, Japan, "Nuclear Transfer in Cattle." 1991.

The American Society of Animal Science. Laramie, WY, "Embryo Sexing: A review of current techniques and their potential for commercial application in livestock production." 1991.

American Red Cross. Rockville, MD, "Production of Transgenic Cattle." 1990.

Texas Tech University. Lubbock, TX, "Embryo Technologies in Animal Production." 1990.

The 1990 Mountain Lake Virginia Workshop Addressing Transgenic Animals as Bioreactors for Therapeutic Proteins. Sponsored by: National Science Foundation and Virginia Polytechnic Institute and State University, Mountain Lake, VA, "Production of Transgenic Cattle." 1990.

Symposium on Advanced Topics in Animal Reproduction. UNESP, Jaboticabal, Brazil, "Production of Identical Bovine Offspring by Nuclear Transfer." 1990.

International Embryo Transfer Society - Annual Meeting. Denver, CO, "Production of Identical Bovine Offspring by Nuclear Transfer." 1990.

The 1989 Mountain Lake Virginia Workshop and Symposia Addressing Transgenic Animals as Bioreactors for Therapeutic Proteins. Sponsored by: National Science Foundation and Virginia Polytechnic Institute and State University, Mountain Lake, VA, "Production of Transgenic Cattle." 1989.

AgBiotech '89 - International Conference & Exposition. Arlington, VA. "Production of Transgenic Cattle by Pronuclear Injection." 1989.

International Embryo Transfer Society - Annual Meeting. San Diego, CA, "The Use of Male-Specific Chromosomal DNA Fragments to Determine the Sex of Bovine Preimplantation Embryos." 1989.

Symposium on Transgenic Technology in Medicine and Agriculture. Sponsored by: Center for Population Research, National Institute of Child Health and Human Development held at the National Institutes of Health, Bethesda, MD, "Production of Transgenic Cattle by Pronuclear Injection." 1988.

American Embryo Transfer Society - Annual Meeting. Reno, Nevada, "The Effect of Trypsin Washing on Post Thaw Viability of Bovine Embryos." 1988.

Genetic Engineering in Animals - Seminar Program. University of Illinois, Champaign, IL, "Nuclear Transfer and Transgenic Cattle." 1988.

Symposium on Application of Egg and Embryo Technologies to Domestic Animals. Copenhagen, Denmark, "Determination of the Sex of Bovine Embryos by DNA Hybridization." 1987.

Texas A&M Experimental Station Annual Staff Meeting. Physiology Group. College Station, TX, "Commercial Opportunities of Biotechnology in Agriculture." 1987.

PROFESSIONAL ACTIVITIES

- Co-Chairman, program of IETS annual meeting 1996
- Program Chairman, IETS Symposium, 1991.
- NIH "Site Review", 1990.
- Adjunct Associate Professor, Texas A&M University, 1986-1993.
- Reviewer International Embryo Transfer Society Meeting, 1988-1995.
- Ad hoc reviewer for USDA Competitive Research Grants Program, 1989-1995.
- Reviewer for Biology of Reproduction, 1990-1995.
- Reviewer for Theriogenology, 1991-1995.
- Reviewer for Molecular Reproduction and Development, 1991-1995.

PUBLICATIONS

1. Chapters In Books

Bondioli, K.R. and Wall, R.J. Transgenic livestock. In: Altman, A. (ed.) Biotechnology in Agriculture. Marcell Dekker, Inc. New York,NY. In Press.

Bondioli, K.R., Biery, K.A., Hill, K.G., Jones, K.B., De Mayo, F.J. Production of transgenic cattle by pronuclear injection. In: First, N. and Haseltine, F. (eds.) Transgenic Animals. Butterworth-Heinemann, Stoneham, MA, 1991, pp. 265-273.

2. Refereed Journals

Westhusin,M.E., Voelkel, S.A., Moore,K. and **Bondioli, K.R.** 1995. Nuclear transfer in the bovine embryo: A comparison of different methods for in vitro embryo culture. Theriogenology Submitted.

Wilson, J.M., Williams, J.D., **Bondioli, K.R.**, Looney, C.R., Westhusin, M.E. and McCalla, D.F. 1995. Comparison of birth weight and growth characteristics of bovine calves produced by nuclear transfer (cloning), embryo transfer and natural mating. Animal Reproduction Science 38:73.

Bondioli, K.R. 1993. Nuclear transfer in cattle. Mol. Reprod. & Dev. 36:274.

Barnes, F., Endebrock, M., Looney, C., Powell, R., Westhusin, M., and **Bondioli, K.** 1993. Embryo cloning in cattle: The use of in vitro matured oocytes. J. Reprod. Fert. 97:317.

Moore, K. and **Bondioli, K.R.** 1993. Glycine and alanine supplementation of culture medium enhances development of bovine in vitro matured and fertilized embryos. Biol. Reprod. 48:833.

Wilson, J.M., Jones, A.L., Moore, K., Looney, C.R. and **Bondioli, K.R**. 1993. Superovulation with a recombinant DNA bovine stimulating hormone. Anim. Reprod. Sci., 33:71.

Westhusin, M.E., Levanduski, M.L. and **Bondioli, K.R.** 1992. Viable embryos and normal calves following nuclear transfer into Hoechst stained enucleated bovine demi-oocytes. J. Reprod. Fert. 95:475.

Bondioli, K.R. 1992. Embryo Sexing: A review of current techniques and their potential for commercial application in livestock production. J. Anim. Sci. Vol. 70 Supplement 2:19.

Wilson, J.M., Zalesky, D.D., Looney, C.R., **Bondioli, K.R.**, and Magness, R.R. 1992. Hormone Secretion by Preimplantation Embryos in a Dynamic In Vitro Culture System. Biol. Reprod. 46:295.

Gray, K.R., **Bondioli, K.R**. and Betts, C.L. 1991. The commercial application of embryo splitting in beef cattle. Theriogenology 35:37.

Refereed Journals (Cont'd):

Westhusin, M.E., Pryor, J.H. and **Bondioli, K.R. 1991**. Nuclear transfer in the bovine embryo: A comparison of 5-day, 6-day, frozen-thawed, and nuclear transfer donor embryos. Mol. Reprod. & Dev. 28:119.

Bondioli, K.R., Westhusin, M.E. and Looney, C.R. 1990. Production of identical bovine offspring by nuclear transfer. Theriogenology 33:165.

Bondioli, K.R., Ellis, S.B, Pryor, J.H, Williams, M.W., and Harpold, M.M., 1989. The use of male-specific chromosomal DNA fragments to determine the sex of bovine preimplantation embryos. Theriogenology 31:95.

Nieman, H., Pryor, J.H., and **Bondioli, K.R.**, 1987. Effects of splitting the zona pellucida and its sealing on freeze-thaw survival of day-7 bovine embryos. Theriogenology: 28:675.

Templeton, J.W., Tipton, R.G., Garber, T., **Bondioli, K.R.**, and Kraemer, D.C., 1987. Expression and genetic segregation of parental BoLA sero types in bovine embryos. Animal Genetics: 18:317.

Merry, D.A., **Bondioli, K.R.**, Allen, R.L., and Wright, R.W., Jr, 1984. One-step sucrose dilution of frozen-thawed sheep embryos. Theriogenology 22:433.

Bondioli, K.R. and Wright, R.W., Jr., 1983. In vitro fertilization of ovulated and ovarian oocytes. J. Anim. Sci. 57:1006.

Bondioli, K.R. and Wright, R.W. Jr., 1983. In vitro fertilization of bovine oocytes by spermatozoa capacitated In vitro. J. Anim. Sci. 57:1001.

Loewus, M.W., Wright, R.W., Jr., **Bondioli, K.R.**, Bedgard, D.L., and Karl, A., 1983. Activity of myo-inositol-1-phosphate synthase in the epididymal spermatozoa of rams. J. Reprod. Fert. 69:215.

Bondioli, K.R., Allen, R.L., and Wright, R.W. Jr., 1982. Induction of estrus and superovulation in seasonally anestrous ewes. Theriogenology 18:209.

Allen, R.W., **Bondioli, K.R,** and Wright, R.W. Jr., 1982. The ability of fetal calf serum, new-born calf serum and normal steer serum to promote the in vitro development of the bovine morulae. Theriogenology 18:185.

Fredericks, G.R., Kincaid, R.L., Wright, R.W. Jr., and **Bondioli, K.R.**, 1981. Effects of dietary coumestrol
on reproduction in mice. Proc. Soc. Exp. Bio. Med. 167:237.

Wright, R.W. Jr., and **Bondioli, K.R.**, 1981. Various aspects of in vitro fertilization and embryo culture in farm animals. J. Anim. Sci. 53:702.

Wright, R.W. Jr., Grammer, J., **Bondioli, K.R.**, Kuzan, F., and Menino, A.R. Jr., 1981. Protein content of porcine embryos during the first nine days of development. Theriogenology 15:235.

Refereed Journals (Cont'd):

Wright, R.W. Jr., **Bondioli, K.R.**, Grammer, J., Kuzan, F., and Menino, A.R. Jr., 1981. FSH or FSH + LH superovulation in ewes following estrus synchronization with medoxyprogesterone acetate pessaries. J. Anim. Sci. 53:115.

Bondioli, K.R., and Wright, R.W. Jr., 1980. Influence of culture media on in vitro fertilization of ovulated ovine oocytes. J. Anim. Sci. 41:660.

2. Abstracts

Bondioli, K.R. and Wall, R.J. 1996. Positive selection of transgenic bovine embryos in culture. Theriogenology 45:345.

Powell, AM, **Bondioli, KR**, and Rexroad, CE Jr. 1996. The sheep uterus as host for IVM-IVF bovine embryos form day 7 - day 15. Theriogenology 45:217.

Bondioli, K.R., Hawk, HW and Wall, RJ. 1995. Effect of glycine and alanine on co-culture of bovine blastocysts. Theriogenology 43:170.

Hill, K.G., Curry, J., DeMayo, F.J., Jones-Diller, K., Slapak, J.R., and **Bondioli, K.R.** 1992. Production of transgenic cattle by pronuclear injection. Theriogenology 37:222.

Voelkel, S.A., Hu, Y.X., Moore, K., and **Bondioli, K.R.** 1992. Freeze survival of bovine embryos produced by in vitro maturation, fertilization and culture of oocytes. Theriogenology 37:317.

Moore, K. and **Bondioli, K.R.** 1991. Amino acid supplementation of culture medium enhances development of bovine IVM/IVF embryos. Serono Symposia, USA.

Moore, K. and **Bondioli, K.R**. 1990. Altering metabolic substrates of culture media enhances bovine IVM/IVF embryo development beyond the eight-cell block. Biol. Reprod. 42 Suppl. 1:55.

Westhusin, M.E., Levanduski, M.J., Scarborough, R., Looney, C.R. and **Bondioli, K.R**. 1990. Utilization of fluorescent staining to identify enucleated demi-oocytes for utilization in bovine nuclear transfer. Biol. Reprod. 42(1):176.

Wolfe, B.A., Westhusin, M.E., Levanduski, M.J., **Bondioli, K.R.** and Kraemer, D.C. 1990. Preimplantation development of embryos produced by intergeneric nuclear transplantation. Theriogenology 33:350.

Jackson, S.P., Forrest, D.W., **Bondioli, K.R**. and Looney, C.R. 1989. Selection of recipient females by ultrasonic imaging of the bovine ovary. J. Anim. Sci. 67 Suppl. 1/J. Dairy Sci. 72 Suppl. 1:355.

Abstracts (Cont'd):

Jones, A.L., Looney, C.R., **Bondioli, K.R**. and Moore, K. 1989. Effect of follicle stimulating hormone (FSH-P) or human chorionic gonadotrophin (hCG) treatment on day three of the estrous cycle on subsequent superovulation. J. Anim. Sci. 67 Suppl. 2:62.

Looney, C.R., Westhusin, M.E., **Bondioli, K.R**. 1989. Effect of cooling temperatures on pre-compacted bovine embryos. Theriogenology 31:218.

Wilson, J.M., Zalesky, D.D., **Bondioli, K.R**. and Looney, C.R., 1988. Hormone secretion by bovine embryos in a dynamic in vitro culture system. Biol. Reprod. 38:Supplement 1:164.

Biery, K.A., **Bondioli, K.R.**, and De Mayo, F.J., 1988. Gene transfer by pronuclear injection in the bovine. Theriogenology. 29:224

Chappel, S., Looney, C.R., and **Bondioli, K.R.**, 1988. Bovine FSH produced by recombinant DNA technology. Theriogenology. 29:235

Ellis, S.B., **Bondioli, K.R.**, Williams, M.E., Pryor, J.H., and Harpold, M.M., 1988. Sex determination of bovine embryos using male-specific DNA probes. Theriogenology, 29:242.

Looney, C.R., **Bondioli, K.R.**, Hill, K.G., and Massey, J.M., 1988. Superovulation of donor cows with bovine follicle-stimulating hormone (bFSH) produced by recombinant DNA technology. Theriogenology, 29:271.

Sasser, R.G., Ruder, C.A., **Bondioli, K.R**., and Hill, K.G., 1987. Pregnancy specific protein B (PSPB) in sera of cows recipient of transferred embryos. American Society of Animal Science, Western Section, Logan Utah.

Bondioli, K.R., and Hill, K.G., 1986. The effect of exposing media to syringes on the viability of bovine embryos. Twelfth Annual Conference of the International Embryo Transfer Society. Colorado Springs, CO. Theriogenology 25:142.

Hill, K.G., **Bondioli, K.R.**, and Looney, C.R., 1986. Use of a norgestomet implant in conjunction with follicle stimulating hormone (FSH) for superovulation of donor cattle. Twelfth Annual Conference of the International Embryo Transfer Society. Colorado Springs, CO. Theriogenology 25:160.

Miller, A.M., Procknor, M., Zalesky, D.D., Thayer, K.M., Forrest, D.W., **Bondioli, K.R.**, Looney, C.R., Hill, K.G., and Welsh, T.H., Jr., 1986. Plasma levels of insulin-like growth factor-I/somatomedin C in superovulated cows. Twelfth Annual Conference of the International Embryo Transfer Society. Colorado Springs, CO. Theriogenology 25:174.

Abstracts (Cont'd):

Zalesky, D.D., Thayer, K.M., Forrest, D.W., Welsh, T.H., Jr., **Bondioli, K.R.**, Looney, C.R., and Hill, K.G., 1986. Relationships between endocrine and ultrasound evaluation of ovulation in superovulated cows. Twelfth Annual Conference of the International Embryo Transfer Society. Colorado Springs, CO. Theriogenology 25:220.

Bondioli, K.R., and Mertes, P.C., 1985. The effect of Ca levels in the freezing media on in vitro survival of bovine embryos. Eleventh Annual Conference of the International Embryo Transfer Society. Montreal, Quebec. Theriogenology 23:181.

Looney, C.R., **Bondioli, K.R.**, Roach, R.T., Oden, A.J., and Massey, J.M., 1985. Prostaglandin F (PGF) treatments for luteal regression in superovulation regimens of donor cattle. Eleventh Annual Conference of the International Embryo Transfer Society. Montreal, Quebec. Theriogenology 23:206.

Mertes, P.C., and **Bondioli, K.R.**, 1985. Effect of splitting technique on pregnancy rate from half embryos. Eleventh Annual Conference of the International Embryo Transfer Society. Montreal, Quebec. Theriogenology 23:209.

Bondioli, K.R., Brunson, C.B., Looney, C.R., Massey, J.M., McGrath, A.B., Mertes, P.C., and Oden, A.J., 1984. In vitro survival of bovine embryos frozen in media supplemented with newborn calf serum or bovine serum albumin. Tenth Annual Conference of the International Embryo Transfer Society. San Antonio, TX. Theriogenology 21:223.

Merry, D.A., **Bondioli, K.R.**, Allen, R.L., and Wright, R.W., Jr., 1983. One-step sucrose dilution of frozen-thawed sheep embryos. American Society of Animal Science. 73rd Annual Meeting, Pullman, WA.

Bondioli, K.R., and Wright, R.W., Jr.,1982. In vitro fertilization of bovine oocytes by spermatozoa capacitated in vitro. American Society of Animal Science 74th Annual Meeting, Guelph, Ontario.

Allen, R.L., **Bondioli, K.R.**, and Wright, R.W., Jr., 1982. The ability of fetal calf serum, newborn calf serum, and normal steer serum to promote in vitro development of bovine morulae. Eighth International Congress on Embryo Transfer. Denver, CO. Theriogenology 17:73.

Allen, R.L., **Bondioli, K.R.**, and Wright, R.W., Jr., 1982. Induction of estrus and superovulation in seasonally anestrous ewes. Eighth International Congress on Embryo Transfer. Denver, CO. Theriogenology 17:74.

Bondioli, K.R., and Wright, R.W., Jr., 1982. In vitro fertilization of ovine ovulated and ovarian oocytes. Eighth International Congress on Embryo Transfer. Denver, CO. Theriogenology 17:79.

Abstracts (Cont'd):

Bondioli, K.R., and Wright, R.W., Jr., 1981. Superovulation of goats with FSH or FSH + LH following estrus synchronization. Seventh International Congress on Embryo Transfer. Denver, CO. Theriogenology 15:118.

Fredericks, G.R., Kincaid, R.L., Wright, R.W., Jr., and **Bondioli, K.R.**, 1980. Effect of low levels of dietary coumestrol on mouse reproductive performance. American Society of Animal Sciences, 72nd Annual Meeting, Ithaca, NY. Abstract 423.

Wright, R.W., Jr., **Bondioli, K.R.**, Grammer, J.C., Kuzan, F.B., and Menino, A.R., Jr., 1980. Superovulation in ewes following estrus synchronization with medoxyprogesterone acetate pessaries. American Society of Animal Science. 72nd Annual Meeting, Ithaca, NY. Abstract 563.

Bondioli, K.R., and Wright, R.W., Jr., 1980. Superovulation of progestogen-synchronized ewes. 6th International Congress on Embryo Transfer. Denver, CO. Theriogenology 13:89.

Fredericks, G.R., Kincaid, R.L., Wright, R.W., Jr., and **Bondioli, K.R.**, 1979. Effects of dietary estrogens on reproduction in the mouse. 74th Annual Meeting, American Dairy Science Association. Logan, UT. J. Dairy Sci. 63:173.

Bondioli, K.R. and Wright, R.W., Jr., 1979. Fertilization in vitro of ovine oocytes. 71st Annual Meeting, American Society of Animal Science. Tucson, AZ. Abstract 345.

B

GENE TRANSFER BY PRONUCLEAR INJECTION IN THE BOVINE

K.A. Biery*, K.R. Bondioli and F.J. De Mayo**

Granada Genetics, Inc.
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**Department of Cell Biology
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Transgenic 60-day bovine fetuses were produced by pronuclear injection of ova with a linear construct of chloramphenicol acetyltransferase structural gene fused to a Rous sarcoma viral promotor (RSVCAT). Ova were collected from the excised oviducts of donors which had been superovulated with 8 injections of follicle stimulating hormone (FSH) over a 5 day period. Prostaglandin (25 mg) was given with the 5th and 6th FSH injections and 4000 IU of hCG was administered at noon or in the evening of day 5 of the injections. The cows were artificially inseminated in the morning and evening of day 6 and ova were recovered between 8:00 am and 1:00 pm on day 7. Dulbecco's modified phosphate buffered saline with 0.4% bovine serum albumin and 1% penicillin (100 units/ml)/streptomycin (100 ug/ml) was the medium used for both the collection and holding of ova. A total of 1,325 ova were recovered and centrifuged at 15,000 x g for 4 min. to aid in the visualization of the pronuclei. Of those collected, 819 embryos (62%) contained visible pronuclei or nuclei which were subsequently injected with the RSVCAT gene. The injected zygotes were transferred (either with or without imbedding in double layer agar chips) into ewes whose oviducts had been ligated at the uterotubal junction. These ewes were either pregnant or under the influence of 1/3 of a Syncro-Mate B implant. throughout this in vivo culture period. Ova were collected from the ewe's oviduct in situ 6 days following transfer with an overall recovery rate of 84%. Of all ova recovered from the sheep, 175 (21% of those injected) developed into morulae or blastocysts and were nonsurgically transferred into synchronized bovine recipients. There was a total of 79 fetuses recovered (10% of those injected) by flank incisions at approximately 60 days of gestation. All fetuses and placentas were analyzed by Southern blot analysis.

For the first phase of the experiment, 459 of the previously described zygotes were injected with 2 ng/ul of the RSVCAT construct. Of those injected, 93 viable embryos were recovered from the ewes and transferred into bovine recipients. There were 39 fetuses recovered at 60 days of gestation (8.2% of those injected) and, in one case, both the fetus and placenta were found to have incorporated the RSVCAT gene (0.22% of those injected).

In the second and subsequent phase of the experiment, ova with visible pronuclei or nuclei from each donor were divided into three equal groups for use in a titration study (120 embryos injected per treatment). These embryos were then injected with either 1, 2 or 4 ng/ul of the RSVCAT construct and analyzed for incorporation. These treatments produced pregnancy rates of 10, 11 and 10% (per embryo injected), respectively. Of all embryos injected with the RSVCAT gene, only one embryo injected with 1 ng/ul and two of the embryos injected with 2 ng/ul produced transgenic fetuses. These data indicate a 0.83%, 1.67% and 0% incorporation rate per embryo injected in the respective treatment groups. Both the fetus and placenta were found to be transgenic in all three cases, and CAT activity was observed in one of the placental samples confirming expression of the RSVCAT gene. These data indicated that, although inefficient under the stated conditions, the RSVCAT gene can be successfully incorporated and expressed in the bovine genome.

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CHAPTER
22

Production of Transgenic Cattle by Pronuclear Injection

Kenneth R. Bondioli
Karen A. Biery
Keith G. Hill
Karen B. Jones
Franko J. De Mayo

Pronuclear injection of one-cell embryos has been used extensively to produce transgenic mice and, to a lesser extent, rabbits, pigs, and sheep. One-cell bovine embryos were injected with a pRSVCAT construct (see below) and fetal tissues were collected for analysis of integration and expression. Integration was found to have occurred in seven fetal samples and expression of the transgene was observed in placental tissue of one sample.

Germline genetic transformation of laboratory mice was first reported by Gordon et al. (1980). Reports from several other laboratories followed quickly, establishing the fact that cloned, foreign DNA would be stably integrated into the genome and that Mendelian germline transmission occurred after microinjection into the pronuclei of fertilized mouse embryos

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(Brinster et al. 1981; Costantini and Lacy 1981; Gordon and Ruddle 1981; Wagner et al. 1981).

The method of choice for the transfer of cloned DNA into mammalian embryos is pronuclear injection. The technique for pronuclear injection of mouse embryos has been described by several authors (Gordon et al. 1980; Hogan et al. 1986; Brinster et al. 1985; Palmiter and Brinster 1986; Hammer et al. 1986; Murray et al. 1988). The production of transgenic rabbits, pigs, sheep, and cattle by pronuclear injection has also been reported (Hammer et al. 1985 and 1986; Brem et al. 1986; Church et al. 1986; Ward et al. 1986; Nancarrow et al. 1987; Simons et al. 1988; Biery et al. 1988).

A number of variables that affect the success rate of pronuclear injection have been studied by Brinster et al. (1985). Walton et al. (1987) have studied specific variables associated with pronuclear injection that affect the lysis of mouse and sheep embryos. The production of transgenic cattle by pronuclear injection has been reported by two laboratories (Church et al. 1986; Biery et al. 1988). In this chapter we describe our experiences with the production of transgenic cattle by pronuclear injection. Some of these data have been previously reported in abstract form (Biery et al. 1988).

22.1 MATERIALS AND METHODS

22.1.1 Collection of Embryos

One-cell bovine embryos were collected from excised oviducts of Holstein and cross-bred, beef-type donors after induction of superovulation by injections of follicle-stimulating hormone (FSH). The superovulation regimen consisted of eight injections of FSH (FSH-P, Schering) at 12-hour intervals over a five-day period (treatment with FSH was initiated on the evening of day 1). A 25-mg dose of prostaglandin F2f (Lutalyse, Upjohn) was given with the fifth and sixth injections of FSH and 4,000 IU of human chorionic gonadotropin (HCG, Butler) was administered at noon or during the evening of day 5 (44 or 48 hours after the first injection of prostaglandin). The cows were artificially inseminated on the morning and evening of day 6, and ova were collected during the morning of day 7 (36 to 40 hours after administration of HCG). Ova were recovered after surgical removal of the ovaries and oviducts by flushing the excised oviducts with Dulbecco's modified phosphate-buffered saline supplemented with 0.4% bovine serum albumin, 100 units/ml penicillin and 100 μg/ml streptomycin. Ova remained in this medium throughout the injection procedure.

22.1.2 Pronuclear Injection

One-cell embryos were centrifuged at 15,000 $\times$ g to aid in the visualization of pronuclei (Wall et al. 1985). Injections were performed under an inverted microscope (Zeiss or Nikon) equipped with Nomarski optics at 400$\times$ mag-

nification. Embryos were placed in a drop of 3–5 μl of medium on a glass depression slide. A 2-μl drop of diluted DNA was placed next to this drop and both drops were covered with paraffin oil. Manipulations were carried out with the aid of Leitz or Narishige micromanipulators. Embryos were held by a fine, polished, holding pipet (outer diameter approximately 150 μm) and the plasma and nuclear membranes were penetrated with an injection pipet (tip diameter approximately 1–2 μm). DNA was injected with the aid of an oil-filled microsyringe (Stolting) until expansion of the pronucleus was visible (25% expansion or less). In the majority of embryos, the most visible pronucleus was injected. If both pronuclei were equally visible, the larger pronucleus was injected.

22.1.3 Preparation of DNA

The plasmid pRSVCAT, containing the bacterial gene for chloramphenicol acetyltransferase (CAT) under the control of the Rous sarcoma virus long terminal repeat (Gorman et al. 1982a), was linearized by digestion with restriction endonuclease *NdeI* (New England Bio Labs). The DNA fragment was then purified by extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with ethanol. The purified fragment was resuspended in 10 mM Tris (pH 7.5), 0.25 mM EDTA. The concentration of the DNA was then determined spectrophotometrically, and the appropriate concentrations of DNA were made. The DNA was diluted for injection in 10 mM Tris (pH 7.5), 0.25 mM EDTA. The majority of the embryos were injected with DNA at a concentration of 2 ng/μl. In three separate experiments the concentration of injected DNA was varied to determine the effect of concentration of DNA on the viability of embryos and the rates of incorporation.

In the first experiment, concentrations of DNA of 1, 2, and 4 ng/μl were injected. In the second experiment, 2, 25, and 50 ng/μl were injected, and in the third experiment, 2, 50, and 100 ng/μl of DNA were injected. Within each experiment different embryo donors and different injection technicians were equally represented in all treatments. The three experiments were conducted at different times, and, therefore, embryo donors and injection technicians varied between the experiments.

22.1.4 Culture and Transfer of Embryos

After pronuclear injection all embryos were cultured to the morula or blastocyst stage. Culture from the one-cell to the morula or blastocyst stage was conducted either in the ligated sheep oviduct or in vitro. Those embryos cultured in the sheep oviduct were transferred (either with or without embedding in double-layer cylinders of agar) into ewes whose oviducts had been ligated at the uterotubal junction according to procedures described by Willadsen (1979). Embryos remained in the sheep oviduct for six days,

at which time they were flushed from the oviducts and evaluated for development to the morula or blastocyst stage. Those embryos cultured in vitro were placed in 20-μl drops that consisted of a 1:1 mixture of Brinster's Mouse Ovum Culture Medium (BMOC) III medium (Brinster 1972) and bovine oviductal fluid. Two to three microliters of bovine oviductal epithelial cells were added to each drop and drops were covered with silicon oil (Dow Corning).

Embryos were cultured at 38°C in an atmosphere of 5% CO_2 and 95% air for seven days and evaluated for development to the morula or blastocyst stage. In experiments where the effect of various concentrations of DNA was investigated, the method of culture was the same for all treatments.

All embryos that developed to the morula or blastocyst stage were transferred by a nonsurgical technique to synchronous recipients (the age of embryos was assumed to be seven days in all cases). In most cases only one embryo was transferred to each recipient but in a few cases two or three embryos were transferred to a single recipient in the same uterine horn.

22.1.5 Collection and Analysis of Tissue

Established pregnancies were carried to 60 to 65 days of gestation, at which time fetuses were surgically removed with a sample of placental tissue.

Fetal and placental tissue were separated and both were snap-frozen in liquid nitrogen. Tissue remained frozen until analysis for incorporation and expression of the transgene.

Fetal and placental tissue was homogenized in STE [0.15 M NaCl, 20 mM Tris (pH 7.8), 10 mM EDTA, that contained 1% sodium dodecylsulfate and 0.2 mg/ml proteinase K (Boehringer Mannheim Biochemicals)]. The homogenate was incubated at 37°C overnight. DNA was then purified by extraction of the homogenate with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), followed by precipitation with ethanol. The DNA was resuspended in STE and subjected to a second precipitation. The DNA was finally resuspended in 10 mM Tris (pH 7.8), 1 mM EDTA.

In order to check for the presence of pRSVCAT in the bovine DNA, the isolated DNA was subjected to Southern blot analysis (Southern, 1975). Ten micrograms of bovine DNA was digested with restriction endonuclease *Eco*RI (Boehringer Mannheim Biochemicals) and separated by agarose gel electrophoresis. The DNA was then transferred to nitrocellulose paper and probed with ^{32}P-labeled pRSVCAT. The pRSVCAT was labeled using a nick-translation kit (Betheseda Research Laboratories). Bovine DNA isolated from uterine tissue served as the negative control. pRSVCAT was added to bovine DNA at one copy and 10 copies per haploid genome to serve as positive controls.

Digestion of pRSVCAT with *Eco*RI should yield fragments of 2.5, 2.1, and 0.34 kb in size. If the *Nde*I digest of pRSVCAT DNA was integrated as a head-to-tail concatamer, this pattern should be observed. If the trans-

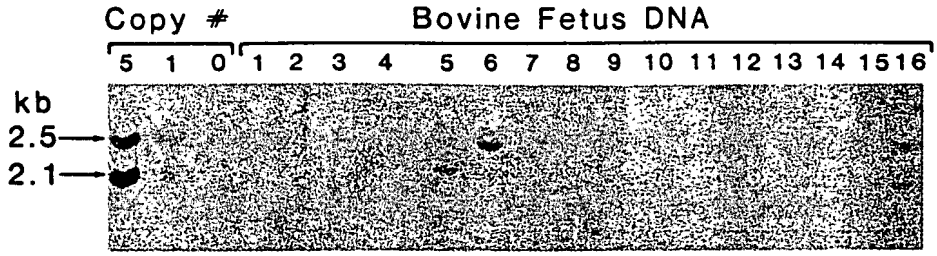

FIGURE 22–1 Southern blot of bovine fetal DNA. Southern analysis was performed as described in the text. Three lanes on the left were loaded with control bovine DNA with pRSVCAT added at the copy number indicated. Fetal samples in lanes 5, 6, and 16 demonstrate integration of the pRSVCAT construct.

gene was integrated as a single copy, only the 2.1- and 0.34-kb bands would be expected. All integrations of bovine DNA observed were of multiple copies. The 0.34-kb fragment was too small to be detected by our blotting procedure (Figure 22–1).

In order to examine expression of the transgene, fetal limb and placental tissues from four positive and two negative samples were assayed for the presence of CAT activity. The tissues were homogenized in 0.25 M Tris (pH 7.5) at 4°C in a Brinkman Polytron. The homogenates were denatured at 65°C for 5 minutes and centrifuged for 10 minutes in an Eppendorf microfuge. The concentrations of protein in the supernatant were determined with a Biorad Protein Assay kit by the Bradford-Lowry procedure. CAT activity was determined in aliquots of supernatant that contained 160 μg of total protein (Gorman et al. 1982b). The assay was incubated at 37° C overnight with 0.5 μCi dichloroacetyl 1-1-2-^{14}C-chloramphenicol, (50 mCi/mmol) (New England Nuclear) and 1.3 mM acetylcoenzyme A. Samples were placed on a thin-layer chromatography (TLC) plate and chromatographed in a 19:1 mixture of chloroform and methanol. The TLC plate was then dried and exposed to X-ray film.

22.2 RESULTS AND DISCUSSION

The procedures for superovulation and collection described above yield approximately 10 ova per collection, of which approximately 70% are fertilized (data not shown). Approximately 90% of the ova collected were one-cell embryos with the remaining 10% being two- to four-cell embryos. Results for embryos injected with the pRSVCAT construct are presented in Table 22–1.

The difference between the proportion of embryos that were fertilized and the proportion of embryos injected (70% fertilized versus 55% injected) can be attributed primarily to the collection of very late pronuclear embryos

TABLE 22–1 Pronuclear Injection of Bovine Embryos with the RSVCAT Construct

No. of ova collected	6207
No. of ova injected	3398 (55%)
No. of ova developing	561 (17%)
No. of fetuses collected	206 (37%)
No. of transgenic fetuses	7 (3.4%)

in which nuclear membranes have started to break down prior to pronuclear fusion.

Microinjection of bovine embryos greatly reduces their viability. Nonmanipulated one-cell embryos cultured in either the sheep oviduct or the culture system in vitro, as described above, develop to the morula or blastocyst stage at a rate of 50–60% (data not shown). This reduction in survival of embryos is similar to that observed for microinjected embryos of pig and sheep (Hammer et al. 1986; Rexroad and Wall 1987; Rexroad and Pursel 1988).

The overall efficiency of integration (seven transgenics from 3398 injected embryos equals 0.2%) is considerably lower than that typically obtained with mouse embryos (Brinster et al. 1981; Brinster et al. 1985) and is somewhat lower than that observed with sheep (Hammer et al. 1985; Nancarrow et al. 1987; Murray et al. 1988; Rexroad and Wall 1987) and pigs (Brem et al. 1986; Hammer et al. 1985; Rexroad and Pursell 1988). The lower overall efficiency of gene incorporation was due both to the low proportion of injected embryos that produced fetuses (6%) and to the low proportion of collected fetuses in which the transgene was incorporated. The results from the DNA concentration experiments are presented in Table 22–2.

Injection of embryos with concentrations of DNA greater than 2 ng/μl did not increase the rates of integration of the DNA. These results are similar to those obtained by Brinster et al. (1985) with mouse embryos. They reported an increase in integration frequency when concentrations of DNA were increased from 0.01 ng/μl to 1 ng/μl, but rates of integration did not increase with concentrations of DNA of above 1 ng/μl. The results in Table 22–2 show that we obtained a total of four transgenic fetuses. The remaining three transgenic fetuses shown in Table 22–1 were from embryos injected with a concentration of DNA of 2 ng/μl.

Viability of embryos varied considerably between the three experiments, a result that is not surprising since embryo donors and injection technicians differed between the experiments. Within each experiment, however, viability was consistently reduced when concentrations of DNA greater than 4 ng/μl were injected. This observation is also in agreement with those of Brinster et al. (1985).

TABLE 22-2 Effect of the Concentrations of Injected DNA on Survival of Embryos and Frequency of Integration

DNA Concentration	*No. Injected*	*No. Developing*	*(%)*	*No. Fetuses*	*% of Transferred*	*% of Injected*	*No. Transgenic*
Experiment I							
1 ng/μl	120	27	(23)	12	44	10.0	1
2 ng/μl	120	23	(19)	14	61	11.6	2
4 ng/μl	120	21	(18)	11	52	9.2	0
Experiment II							
2 ng/μl	124	21	(17)	7	33	5.6	0
25 ng/μl	118	14	(12)	4	29	3.4	1
50 ng/μl	100	6	(6)	3	50	3.0	0
Experiment III							
2 ng/μl	55	16	(29)	6	38	10.9	0
50 ng/μl	60	11	(18)	3	27	5.0	0
100 ng/μl	52	8	(15)	3	38	5.7	0

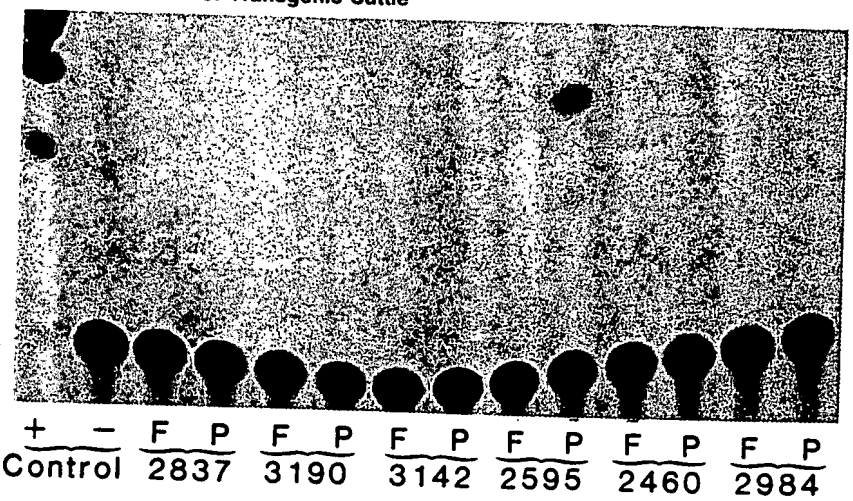

FIGURE 22–2 Autoradiograph of TLC plate from assay of chloramphenicol acetyltransferase (CAT) activity. CAT activity was assayed in fetal and placental tissue as described in the text. Sample number 2595 showed evidence of CAT activity in the placental tissue.

When four of the samples that were positive for integration were assayed for expression of the transgene, one sample (sample no. 2595) showed evidence of expression in the placental tissue (Figure 22–2). No expression was observed in fetal limb tissue from this same sample. The reason for detectable expression in the placental tissue but not in the fetal tissue is unknown.

These results demonstrate that injection of linearized DNA constructs into the pronuclei of bovine embryos can result in integration of the DNA in fetal tissue. This integration occurred without rearrangement of the gene construct and the transgene was capable of being expressed in the placental tissue of one conceptus.

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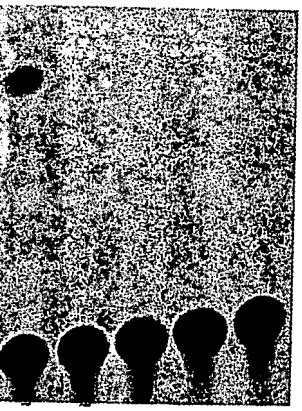

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Risks of Biotechnology

Transgenic Animals

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Animal production industry in the year 2000 A.D.

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Summary. One can easily envision that, in the very near future, all bulls being progeny tested will be screened for genomic markers linked to economic traits and females may also be screened if enrolled as donors in a nuclear transfer programme. The concept of producing large numbers of genetically identical embryos, frozen, sexed, screened for economic traits and produced inexpensively from slaughterhouse by-products, is within our grasp. While large scale commercialization of these concepts is a function of time, knowledge and cost-effective biotechnologies, all of these concepts have already been demonstrated. The production of transgenic animals and embryos will be accelerated as gene mapping links genes to economic traits.

What will happen to protein production when commercial cow herds can be made up of one or more female clone lines mated to bulls of the same clone? The obvious answer is predictability of performance to a magnitude never before achieved in agriculture.

Keywords: transgenic; Y-specific probes; nuclear transplantation; restriction fragment length polymorphism

Introduction

Historically, animal scientists have been quick to take advantage of biotechnologies for the propagation of economically superior genetics. Artificial insemination has been a standard practice for the past 40 years in the dairy industry and has resulted in rapid improvement in milk production and milk components. In the past 5 years, the dairy industry has focussed on the improvement of milk solids such as protein and fats. These improvements through artificial insemination have occurred because of the selection pressure applied to the selection of superior bulls used by the artificial insemination organization. In the past 10 years embryo transfer technology has emerged as a practical method of accelerating genetic improvement by taking advantage of the number of offspring which can be produced from the mating of a superior female and progeny-proven sire. New embryo manipulation techniques are on the horizon in application to livestock improvements. Rapid development in molecular biology has resulted in the opportunity to develop superior animal lines which have been enhanced through insertion of a desirable gene into the genome. These developments have been reported for mice (Gordon *et al.*, 1980), sheep (Hammer *et al.*, 1985), pigs (Hammer *et al.*, 1986) and cattle (Biery *et al.*, 1988). These transgenic animals have been produced by the injection of a unique gene construct such as the human and rat growth hormone (Palmiter *et al.*, 1982, 1983) into early stage developing embryos. The development of molecular genetic markers detecting variations at the DNA level has opened the door to the mapping of not only the human genome but also the mapping of farm animals such as cattle (Womack, 1988). The intent of this paper will be to review the current and developing biotechnologies which will have an impact on the production of animals in agriculture into and beyond the year 2000 A.D.

Embryo transfer

The ability to manipulate embryos at early stages of development opens up a number of possibilities for increasing desirable genotypes in economically important food animals. Research into the techniques of embryo manipulation can also lead to basic knowledge about developmental biology.

The non-surgical recovery of embryos from superovulated donor cows (Elsden *et al.*, 1978) was a major step in the commercialization of embryo transfer technology in the cattle industry. Embryo transfer in both dairy and beef cattle is now an accepted commercial practice, producing a large number of embryo transfer offspring. The techniques have been applied to other food animals such as pigs, sheep, and goats, but acceptance of the procedure has been much slower because recovery of embryos from these species is still by surgical means, thus potentially limiting their reproductive life. The advancement of molecular biology will have an impact on the embryo transfer industry in the future with production of recombinant gonadotrophins for superovulation (Wilson, 1988). When recombinant bovine follicle-stimulating hormone (bFSH) was used to superovulate 205 cows the mean response was 11·1 total ova, of which 85% (9·4) were viable embryos (Table 1). When the best stimulation programme was reported, total ova and viable embryos increased slightly to 12·4 ova and 11·0 (89%) (Table 2) respectively. The means were not different ($P > 0·05$). When a pituitary extract-FSH (FSP-P®) was used for superovulation 10 783 cows produced a mean of 10·32 total ova, of which 57% (5·85) were viable embryos (Table 3). When the best three FSH-P stimulation programmes were reported, total ova and viable embryos increased to 12·16 and 6·72 (55%) (Table 4) respectively. The means were not different ($P > 0·05$). It was also reported that the percentage of Grade 1 and 2 embryos collected was greater for cows stimulated with bFSH than for cows stimulated with FSH-P. The percentages of Grade 1, 2 and 3 embryos were 53%, 39% and 8% respectively for bFSH, with Grade 1 being embryos which were at the proper stage of development and morphology, and embryos of Grades 2 and 3 being of lesser developmental quality. The percentages of Grade 1, 2 and 3 embryos were 39%, 40·3% and 20% respectively for FSH-P. While the total ova of 12·4 for bFSH and 12·16 for FSH-P are essentially the same, the viable embryos of 11·0 and 6·72 for bFSH and FSH-P, respectively, are different ($P < 0·05$) (Duncan multiple range test). There could be several reasons for this difference beside the obvious one that the recombinant bovine FSH should be identical to the endogenous FSH of the cow. Another reason is that bFSH should be free of any other gonadotrophic hormone such as luteinizing hormone (LH). It can be postulated that additional exogenous LH is not needed to superovulate cattle or for ovum maturation.

Table 1. Embryo production from 205 donor cows stimulated with bFSH (May 1987–May 1988)* (data from Wilson, 1988)

	Total ova	Viable embryos	Unfertilized ova†	Degenerate embryos
	2267	1921	162	185
Mean/cow	11·1	9·4	0·8	0·9
%		85·0	7·2	8·1

*Doses ranging from 0·5 mg twice/day to 8·0 mg for 3–5 days.
†Four cows accounted for 60% of the total unfertilized ova.

The sex ratio

Controlling the sex ratio of offspring is of significant commercial value in agriculture and would best be controlled by sexed semen. There have been extensive efforts to produce and occasional

Table 2. Embryo production in 99 cows stimulated with FSH regimens which appear to be most efficacious and cost effective (data from Wilson, 1988)

	Total ova	Viable embryos	Unfertilized ova	Degenerate embryos
	1231	1089	67	75
Mean/cow	12·4	11·0	0·7	0·7
%		89·0	5·6	5·6

Table 3. Embryo production from 10 783 donor cows stimulated with FSH-P (1983–July 1988) (data from Wilson, 1988)

	Total ova	Unfertilized ova	Degenerate embryos	Viable embryos
	111 280	29 653	18 546	63 080
Mean/cow	10·32	2·75	1·72	5·85
%		26	17	57

Table 4. Embryo production from 5979 cows treated with 3 stimulation programmes after utilization of two 25-mg PGF injections (data from Wilson, 1988)

	Total ova	Unfertilized ova	Degenerate embryos	Viable embryos
	72 704	20 089	12 436	40 178
Mean/cow	12·16	3·36	2·08	6·72
%		28	17	55

claims of success in producing a sexed semen product, but today a reproducible technique for separating X- and Y-bearing spermatozoa has not been accomplished for farm animals (but see Johnson *et al.*, 1989). The economic potential of sexed semen is so extensive that it continues to warrant significant effort.

Sex ratio will first be controlled through embryo manipulation before embryo transfer. Cytological approaches to embryo sexing have been reviewed (King, 1984). Embryo splitting allows half the embryo to be karyotyped and the other half used for transfer. The pregnancy rate from split embryos is only slightly less than that for intact embryos. A major limitation is that half embryos are not easily frozen for later transfer, thus reducing the practical value of the approach. Karyotyping of embryos is also limited by the inability to generate mitotic structures sufficient to identify the sex chromsomes consistently due to the small number of cells available; sex determination is only reported 60% of the time.

It has also been reported that male-specific antigens are expressed as early as the 8-cell stage in embryos of mice, cattle, pigs and sheep (Wachtel, 1984) and that they can be identified by indirect immunofluorescence with antisera raised in female mice against spleen cells from males of the same inbred strain. A high degree of success has only been demonstrated in one laboratory (Anderson, 1987). This technique is highly variable and needs further verification.

The use of male-specific chromosomal DNA fragments to determine the sex of cattle embryos has been reported (Bondioli *et al.*, 1989). The identification of repetitive, male-specific chromo-

somal DNA fragments allows use of DNA-probe technology to determine the sex of cattle embryos from a small embryonic sample. These Y chromosome-specific probes can be used in conjunction with DNA-replicating techniques such as a polymerase chain reaction, thus decreasing the number of cells required to sex an embryo, potentially to 1 or 2 cells. The removal of 1 or 2 cells essentially leaves the embryo intact for further manipulation such as embryo freezing. The accuracy of these techniques is essentially 100% with the limitation being a function of sufficient DNA sampling and recombinant DNA techniques.

In-vitro maturation and fertilization of oocytes

At the present time a large demand exists for cattle oocytes and early stage embryos for use in commercial operations and research. Basic research with cattle and other farm animals has been hampered by an inadequate source of competent oocytes and early stage embryos at an economically feasible cost. Research into the current biotechnologies for manipulating the genome of cattle or other farm animals also requires a large supply of oocytes and/or early stage embryos. A system to take advantage of oocytes from ovaries of commercially slaughtered cattle would be the best economic supply. In-vitro produced oocytes or embryos also have the advantage of known timing of development compared to those recovered *in vivo*. In-vitro culture of bovine primary oocytes taken from slaughterhouse ovaries and fertilized *in vitro* have been reported (Critser *et al.*, 1986) to produce live young. Currently variable efficiency exists in producing developmentally competent zygotes from the in-vitro maturation–in-vitro fertilization system (Critser *et al.*, 1986; Lu *et al.*, 1987; Xu *et al.*, 1987; Fukui & Ono, 1988).

A system for large scale oocyte production of slaughterhouse ovaries has been implemented by Granada Genetics, Inc. The laboratory currently receives 400 ovaries per day yielding about 1000 usable oocytes or 2·5 oocytes per ovary. The oocytes are utilized in an in-vitro fertilization programme and a nuclear transplantation programme. Approximately 20% of all oocytes matured and fertilized *in vitro* yield viable embryos, and approximately 50% of the in-vitro matured oocytes can be used as recipient oocytes for nuclear transfer, yielding about 20% viable embryos when fused and cultured for 5 days in a sheep oviduct. These results are generally comparable to in-vivo results but are much more cost effective.

Embryo splitting and nuclear transplantation

Microsurgical techniques developed for basic studies in mammalian embryology using mouse embryos have been applied to large domestic animals to increase the number of offspring with identical genotypes. Separation of blastomeres of early cleavage-stage embryos of cattle, horses, pigs and sheep have resulted in normal development from as little as a quarter of the normal complement of cells (Willadsen, 1979). Normal offspring can be produced by splitting embryos of late morula or early blastocyst stages into halves.

Another approach to multiplying the number of offspring with identical genotypes is by nuclear transplantation. Nuclear transfer involves the transplantation of living nuclei from typically embryonic cells to unfertilized eggs. The early research on vertebrates was performed in amphibians; nuclear material was taken from separated embryonic frog blastomere cells and introduced into enucleated frog oocytes (Briggs & King, 1952). Transplantation of nuclear material in mammals has proved very difficult to achieve, in part due to the microsurgical techniques used to manipulate embryos and eggs. The microsurgical techniques can be destructive to delicate cell structures necessary for later development. An alternative procedure is to deliver the nuclear material to a recipient egg by fusion of an intact cell or karyoplast consisting of a nucleus surrounded by a piece of plasma membrane.

Successful nuclear transplantation and cell fusion was achieved for sheep embryos when individual blastomeres from 8- and 16-cell embryos were used as the nuclear donor into enucleated or nucleated halves of unfertilized eggs (Willadsen,1986). The production of a live nuclear transplanted lamb was a clear demonstration that the nuclear material from at least the 8–16-cell sheep embryo was totipotent. The potential for cloning livestock had not been expected since in mouse experiments transplanted nuclei from 4-cell and older embryos did not support development to the blastocyst stage (McGrath & Solter, 1984). This may point to significant differences in mammalian embryos in the timing of activation of the embryonic genome and the interaction between nuclei and cytoplasm.

Bovine blastomeres from 2–32-cell-stage embryos recovered surgically were demonstrated to be totipotent when fused to a bovine enucleated oocyte (Prather *et al.*, 1987). Blastomeres from 32-cells to at least a compacted morula stage embryo (approximately 64 cells) (Table 5) collected from cattle non-surgically were found to be totipotent and produced live young when transferred to an enucleated oocyte and later a recipient (unpublished data). It has also been demonstrated that deep-frozen donor embryos produce similar results when transplanted into enucleated oocytes (Fig. 1). Embryos that were produced through nuclear transplantation procedures have in turn been used as nuclear donor cells for subsequent fusion to recipient eggs (Fig. 2). The pregnancy ratio for fresh and frozen nuclear transfer embryos has ranged from 0 to 54% (Fig. 3).

Table 5. Production of nuclear transfer cattle embryos by donor embryo cell number (1st and multiple generation procedures)

Donor embryo cell no.	Viable embryos	No. of transfers	No. pregnant	Pregnancy rate (%)
1–10	53	38	1	2·0
11–19	143	74	11	14·8
20–29	190	126	23	18·25
30–39	168	105	24	22·8
40–49	71	48	12	25·0
50–59	15	10	0	0·0
≥60	60	50	14	28·0
Total	700	451	85	18·8

Successful nuclear transfer transplantations and embryo development in farm animals have great implications. These achievements will provide the first opportunity to make large numbers of identical offspring for milk or beef production. Nuclear transfers will produce uniformity in calf crops and even have an impact on changing the sex ratio of a calf crop. Nuclear transplantation will have a great impact on transgenic production of farm animals since a potential transgenic embryo can be mass produced.

Transgenic animals

Technology for manipulating embryos of farm animals has developed primarily because of the techniques developed for the study of embryonic development of laboratory animals. Germ-line transformation of laboratory mice was first reported in 1980 (Gordon *et al.*, 1980). Reports from several other laboratories established that cloned foreign DNA was stably integrated into the genome and Mendelian germ-line transmission occurred following microinjection into the pronuclei of fertilized mouse embryos (Brinster *et al.*, 1981; Costantini & Lacy, 1981; Gordon & Ruddle, 1981; Wagner *et al.*, 1981). The extrapolation of this technology from laboratory animals to farm animals has not been as simple and straightforward as would be expected. The major drawback has been the high cost associated with obtaining a source of early-stage embryos from

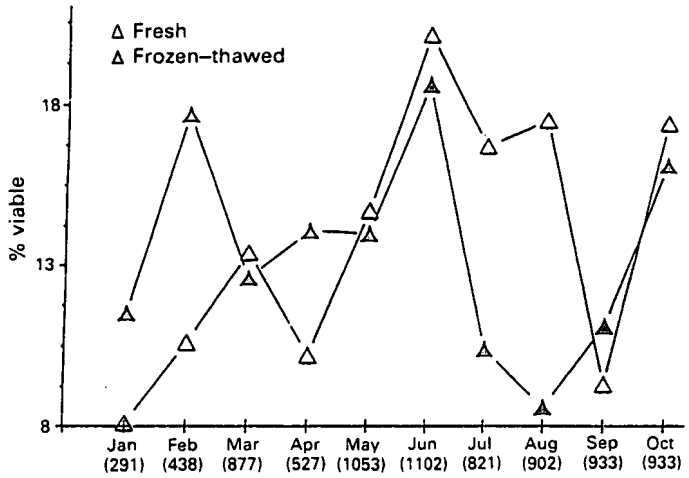

Fig. 1. Percentage production of viable cattle embryos after nuclear transfer. Blastomeres were physically separated from fresh or frozen–thawed embryos and fused (1–15 October only in October) to an enucleated oocyte. The resulting embryos were cultured and evaluated 6 days after fusion. Total no. of fusions given in parentheses.

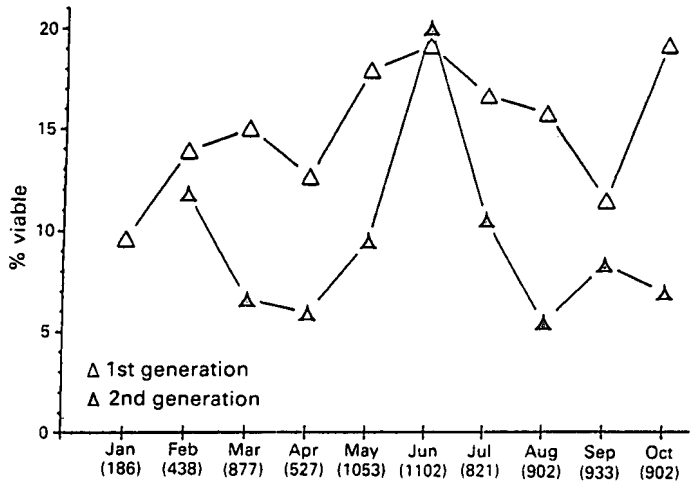

Fig. 2. Percentage production of viable cattle embryos after nuclear transfer. Donor embryos were collected and blastomeres were physically separated and fused to enucleated oocytes (1st generation). The resulting embryos were then used as donor embryos to produce a 2nd generation of embryos by separating blastomeres and fusing to enucleated oocytes. The efficiency was evaluated 6 days after fusion. Total no. of fusions given in parentheses; fusions in October on 1–15 October only.

farm animals and the high cost of maintenance of resulting offspring. The cytoplasm of ova from mice and rabbits is transparent, and the cytoplasm of pig, sheep and cattle ova is opaque, making the pronuclei invisible under light microscopy.

Techniques have been developed for visualizing and injecting pronuclei of sheep, pigs and cattle (Hammer *et al.*, 1985; Wall *et al.*, 1987; Biery *et al.*, 1988). The human growth hormone gene attached to the mouse metallothionein promotor (MT-hGH) has been injected into the pronuclei

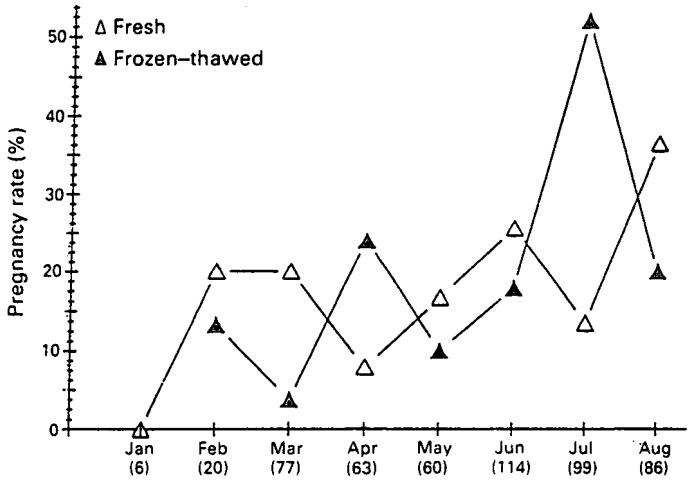

Fig. 3. Pregnancy rate in cows from embryos produced by nuclear transfer. The embryos (no. in parentheses) were transferred to a recipient cow after 6 days of culture of frozen and thawed for later transfer. Pregnancy rate was assessed at 90 days and at birth.

or nuclei of rabbit, pig and sheep ova (Hammer *et al.*, 1985, 1986). Integration of the gene into the DNA of each species was achieved and expression of the gene was observed in rabbits and pigs. The integration frequencies were 12·8% in rabbits, 10·4% in pigs and 1·3% in sheep. The integration frequency of a bacterial chloramphenicol acetyltransferase gene under the control of the Rous sarcoma virus promoter was 3·4% and expression was only observed in the placental tissue of one conceptus.

A system for large scale production of transgenic cattle is currently being evaluated at our laboratory. Bovine pronuclear embryos were collected and injected with a human oestrogen receptor gene linked to a skeletal actin promoter (ASK-HER). The injected embryos were cultured *in vitro* for 7 days before transfer to recipient cattle (Table 6).

The use of in-vitro matured and in-vitro fertilized oocytes is also under evaluation for production of transgenic cattle (Table 7).

Table 6. Production of transgenic cattle with ASK-HER

No. of ova collected	3902
No. of ova injected	1704 (44%)
No. of ova developed in culture	261 (15% of injected)
No. of pregnancies	79 (30% of transferred)
No. of transgenic cattle	1 known from 16 calves born

Tables 6 and 7 clearly demonstrate the efficiency of producing transgenic cattle to be very low but the zygotes produced by in-vitro maturation and fertilization of oocytes are very cost effective when compared to an in-vivo source of pronuclear embryos.

There are other potential methods of integrating genes into the genome of an embryo, such as the utilization of retroviral vectors (King *et al.*, 1985) and embryo-derived stem cells (Evans &

Table 7. Production of transgenic cattle from oocytes matured and fertilized *in vitro*

No. of ova matured	2408
No. of ova injected	858 (36%)
No. of ova developed in culture	49 (6% of injected)
No. of pregnancies	12 (24%)
No. of transgenic calves	Unknown

Kaufman, 1981). Lavitrano *et al.* (1989) reported that when spermatozoa incubated with PSV2CAT plasmid were used to fertilize mouse eggs *in vitro*, integration was observed in approximately 30% of 250 progeny. However, this finding needs to be repeated for mice and tested for other species.

The practicability of transgenic animals for improvement of farm animals cannot be assessed until questions of integration and expression are answered. While many of these questions will probably be answered with experiments on laboratory animals, many will only be answered with the actual production of transgenic farm animals. For example, how might over-expression of a gene like growth hormone affect the complex interplay that regulates growth rate, body composition, overall fitness, age, sexual maturity and reproductive capacity of an animal? Disease resistance genes may be better candidates for transgenic production because they may have fewer physiological repercussions.

The genes of choice

The evidence from large animal studies and theory indicates that single genes, in general, are unlikely to have a significant effect on commercial traits and success will depend on finding the exception, such as the Booroola gene (Land & Wilmut, 1987), which increases litter size in sheep.

Another example of single genes affecting polygenic traits is the double muscling gene of cattle. These genes have disadvantages as well as advantages. The double-muscle gene produces a higher lean yield but also increases calving difficulties. It may be possible to manipulate a number of genes or to manipulate genes that govern a whole cascade of enzymes. The elevated concentration of individual enzymes is unlikely to increase the flux through a complex pathway, but if all enzymes in a pathway are elevated the output would increase proportionally. Research with the pig shows the relevance of this knowledge. Selection for the level of four enzymes (glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, NADP-linked malate dehydrogenase and NADP-linked isocitrate dehydrogenase) in the nicotinamide adenine dinucleotide phosphate (NADP) pathway led to marked changes in backfat (Muller, 1986). After 8 generations of selection, the phenotypes of the high and low lines differ by 3·6 standard deviations. The effectiveness of hormones such as growth hormone or growth hormone-releasing factor was demonstrated in larger transgenic mice (Palmiter *et al.*, 1982; Hammer *et al.*, 1985).

The expression of transgenes at unique physiological age or the expression of site-specific genes would be highly significant. If a growth hormone gene can be linked to a promotor which only expresses at birth and turns off at puberty, it may produce all the desired effects of early growth. Site specific expression of growth hormone in the mammary system may increase milk production without any other physiological effects. Transgenic sheep have been produced with a human anti-haemophilic factor IX gene linked to a sheep derived lactoglobulin promotor (Clark *et al.*, 1989). Two sheep expressed factor IX into their milk, demonstrating that expression can be site specific and that a novel gene can be integrated and expressed in a foreign genome.

Genetic markers

The contribution of recombinant DNA technology may lie in the identification and mapping of polymorphic loci in the genomes of livestock. The identification and mapping of restriction fragment length polymorphisms (RFLPs) have revolutionized human genetics by providing markers for a variety of inherited diseases. Only a few RFLPs in farm animals have been reported (Womack, 1988). If the DNA of domestic animals is as polymorphic as that of humans, one would expect approximately one polymorphic site in every 100 nucleotide pairs (Jeffreys, 1979). The widespread availability of DNA probes and the use of RFLPs for genomic analysis should increase the number of known DNA polymorphisms in farm animals.

The parasexual methods of somatic cell hybridization used extensively in human gene mapping can be applied to any mammalian species (Womack & Moll, 1986; Womack, 1987). Using cow–hamster hybrid cells, 37 bovine isoenzyme loci to 24 syntenic groups have been identified. Gene maps of sheep and pigs are being developed with the same methods at comparable rates.

The number of polymorphic markers required to saturate a mammalian genome depends on the desired density of mapped loci (Womack, 1987). If one assumes a genome size of 2500 cM for a given species, approximately 215 randomly distributed markers are necessary to generate 95% probability that any quantitative-trait locus will be within 20 cM of a polymorphic marker (Kashi *et al.*, 1986). Homology of the human, mouse and cattle maps is extensive (Womack & Moll, 1986); that is, groups of syntenic genes tend to be conserved in all 3 species. This knowledge will allow for the use of human and mouse mapping data to select probes for identification of polymorphisms at loci with predictable spacing along the bovine chromosomes.

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PRODUCTION OF TRANSGENIC CATTLE BY PRONUCLEAR INJECTION

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Previously, this laboratory reported success in producing transgenic bovine fetuses (Biery et al., 1988, Theriogenology 29:224). Since that time, an effort was made to produce live, transgenic cattle. Data for two ova sources and four gene constructs are included. The first ova source was from excised oviducts of cattle stimulated with FSH. These ova were collected at 36 hrs post onset of estrus (24 hr post initial breeding). The second source of ova was IVM-IVF. IVM-IVF ova were subjected to pronuclear injection beginning at 18 hrs post IVF. Structural genes used were human estrogen receptor (HER) and insulin-like growth factor-I (IGF-I). Two promoters from the chicken α-skeletal actin gene were utilized, consisting of 202 bP of the promoter (202-ASK) or the promoter and first intron of the actin gene (733-ASK). Approximately 2 Kb of the mouse mammary tumor virus 3′ long terminal repeat promoter (MMTV) was also used. Injected ova were cultured in an oviductal cell co-culture. At the end of culture, morulae and blastocysts were transferred non-surgically to synchronous recipients. After calving, blood and tissue samples were analyzed for the presence of a transgene by Southern blot analysis.

Construct	Ova Source	No. Ova Collected	No. Ova Injected (%)	No. Ova Developing (%)	No. Preg./ No. Abort	No. Live Calves	No. Transgenic
ASKHER	In-vivo	4150	1878 (45)	266 (14)	82/12	53	1
202-ASK-IGF-I	In-vivo	2668	1346 (50)	205 (15)	62/21	35	1
202-ASK-IGF-I	IVM-IVF	5142	2559 (50)	178[a] (7)	44/8	32	2[b]
MMTV-IGF-I	In-vivo	538	246 (46)	38 (15)	14/5	9	1
MMTV-IGF-I	IVM-IVF	798	667 (84)	63 (9)	15/2	11	1
733-ASK-IGF-I	In-vivo	295	136 (46)	15 (11)	6/4	2	0
733-ASK-IGF-I	IVM-IVF	5775	4374 (76)	293 (7)	92/34	51	1[b]

[a]40 Not transferred.
[b]These calves were stillborn or died within one day of birth.

Ova from IVM-IVF procedures were more synchronous as evidenced by a high proportion of injected ova. In-vivo ova had higher development and pregnancy rates. No gross abnormalities were observed in any calves. Seven animals tested positive for the transgene. The efficiency of producing these transgenic animals (#Transgenic/Ova Injected) ranged from 0.02 to 0.18%. Two bulls lived to sexual maturity. Semen was collected and frozen from these bulls and used to produce embryos through IVM-IVF and co-culture. These embryos were analyzed by PCR for presence of the transgene. Results for a bull with ASKHER showed 34 of 74 embryos to possess the transgene, and for the bull with 202-ASK-IGF-I 64 of 114 embryos possessed the transgene. These data show that transgenic cattle can be produced by pronuclear injection which are capable of transmitting the transgene to progeny.